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Identification and characterization of urokinase receptors in natural killer cells and T-cell-derived lymphokine activated killer cells

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Fluorescence-activated cell scanning analysis of human blood cells revealed novel urokinase receptors in large granular lymphocytes and a small subset of T-cells (CD3+). Culturing of T-cells with interleukin-2 to generate CD3+ lymphokine-activated killer cells caused a large increase in urokinase binding, suggesting that the urokinase receptor is an activation antigen. The receptor in lymphocytes was similar to that in monocytes with regard to size, affinity and ligand specificity, but did not mediate degradation of urokinase-inhibitor complexes. It is suggested that lymphocyte-bound pro-urokinase is activated, e.g. by the human T-cell-specific serine proteinase, HuTSP-1, and thereby starts a cascade of plasminogen activation important for extravasation of the cells.

Urokinase receptor; Lymphocyte; LAK cell; Internalization; Plasminogen activator inhibitor; Activation antigen

1. INTRODUCTION

The plasminogen activator urokinase (uPA) is secreted as a single chain proenzyme (pro-uPA), which binds to uPA receptors (uPAR) of a variety of cells. uPARs are 313 residue glycoproteins [1] largely anchored to the plasma membrane by a C-terminal glycosyl-phosphatidylinositol moiety [2].

Pro-uPA is converted to catalytically active uPA, e.g. by the human T-cell-specific serine proteinase, HuTSP-1, secreted by NK cells and activated T-cells [3,4]. Once a small amount of active uPA has been generated a cascade may be started by activation of the ubiquitous plasminogen to plasmin, which causes further conversion of pro-uPA to uPA [5]. Plasmin degrades several components of the extracellular matrix, and plasminogen activation mediated by receptor-bound uPA is therefore thought to be important for extravasation and invasion of some tumor cells [6].

Plasminogen activation is controlled by inhibitors, e.g. plasminogen activator inhibitor type-1 (PAI-1), which forms a stable complex with uPA. It has been shown that uPA:PAI-1 complexes are rapidly removed from the cell surface by uPAR-mediated endocytosis followed by degradation in the monocytoid cell line, U937 [7], and the choricarcinoma cell line, JAR [8].

Previous work has shown that monocytes [9] and polymorphonuclear leucocytes [10] are uPAR-bearing. This has also been reported for B-cells, whereas T-cells are thought to be uPAR-negative [10]. We have used fluorescence-activated cell scanning (FACS) to analyse the occurrence of uPAR in human lymphocytes. We report hitherto unrecognized uPAR in large granular lymphocytes (LGL), which constitute active NK cells, and interleukin-2 (IL-2)-activated T-cells (LAK cells). In contrast to monocytes, LAK cells did not degrade uPAR-bound uPA:PAI-1 complexes. Recent work has shown that LAK cells, which possess anti-tumor activity, can leave the microcirculation and infiltrate pulmonary metastasis [11]. Our findings suggest the implication of cell surface-organized plasminogen activation in their extravasation.

Abbreviations: DFP, diisopropylfluorophosphate; [^{125}I]DFP-uPA, ^{125}I-labeled and DFP-treated uPA; FACS, fluorescence-activated cell scanning; LAK cells, lymphokine activated killer-cells; LGL, large granular lymphocytes; PAI-1, plasminogen activator inhibitor type-1; PPD, purified protein derivative; uPA, urokinase type plasminogen activator; uPAR, receptor for uPA.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Human recombinant IL-2 was from Amersham, UK. Phosphatidylinositol phospholipase C was from Boehringer-Mannheim, Germany. Human two-chain uPA was from Serono, Switzerland. Pro-uPA (single chain uPA) was purified from serum-free conditioned medium of human HT-1080 cells as described previously [8]. uPA was catalytically inactivated with diisopropylfluoro-phosphate (DFP) as described [9]. uPA and DFP-uPA were iodinated to a specific activity of about 9×10^{15} Bq/mol using chloramin-T as the oxidizing reagent [9].

Biotinylation: 250 μl 80 nM uPA in 0.1 M $NaHCO_3$, pH 9.8, was added to 250 μl 1 mM biotin-*N*-hydroxy-succinamide (Sigma) in dimethylformamide followed by incubation for 1 h at 20°C and dialysis against phosphate-buffered saline, pH 7.2, with 50% glycerol. Biotin-uPA was indistinguishable from uPA and DFP-uPA as inhibitor of binding of [^{125}I]DFP-uPA (cf. Fig. 3).

Human PAI-1 was purified by immunoaffinity chromatography from serum-free conditioned medium of HT-1080 cells cultured with dexamethasone [8]. Labeled uPA:PAI-1 complex was prepared by incubating 1 μg/ml [^{125}I]uPA with 10 μg/ml SDS-activated PAI-1 [12] in 0.1 M Tris, 0.1% Triton X-100, pH 8.1, for 1 h at 20°C followed by passage through anti-PAI-1 IgG and anti-uPA-IgG columns [8]. The murine monoclonal antibody (clone 6) was against the N-terminal part of uPA containing the receptor binding domain [13].

2.2. *Cells*

Human peripheral blood mononuclear cells were prepared from citrate-phosphate-dextrose stabilized buffy coats by flotation on Isopaque-Ficoll. Monocytes were isolated as the population adhering to plastic dishes after incubation for 1.5 h at 37°C [9]. The erythrocyte-rosette-forming fraction (> 95% CD3+) was isolated from the non-adherent cells using sheep red blood cells treated with 1% *S*-(2-aminoethyl) isothiouronium followed by centrifugation on Ficoll, and designated the T-cell fraction. The non-rosetting cells, containing 60–80% CD20+ cells, was designated the B-cell enriched fraction. Polymorphonuclear leucocytes were prepared from buffy coats using Percoll gradient centrifugation as described [14]. LAK cells were prepared by culturing ($1–2 \times 10^6$ cells/ml, 5% CO_2 in atmospheric air) the T-cell fraction in s-RPMI (Gibco, UK) containing 10^3 U/l IL-2. Fresh IL-2 was added every third day and the cells (>95% CD3+, no CD16+) were harvested by about 10 days.

2.3. *FACS analysis*

The cells were incubated with biotin-uPA, washed and resuspended in phosphate buffered saline, pH 7.2, containing streptavidine–phycoerythrinine and FITCH-conjugated cell marker antibodies (Becton Dickinson). After 30 min in the dark, the cells were washed, resuspended and analysed in a FACScan flowcytometer (Becton Dickinson).

2.4. *Binding experiments*

Cells were washed and incubated with labeled and unlabeled ligands in 124 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2.5 mM NaH_2PO_4, 25 mM HEPES, 1% bovine serum albumin, 5 μg/ml Trasylol, pH 7.4. The incubations were terminated by the addition of 250 μl suspension to microfuge tubes containing 200 μl silicone oil, D 1.025, followed by centrifugation [9]. The tube was cut and the pellet assayed for radioactivity. Triton X-114 detergent phase extracts used for blotting of [^{125}I]DFP-uPA (cf. legend to Fig. 3, inset) were prepared as described by Behrendt et al. [15]. In brief, 2×10^8 cells were lysed in 4 ml 0.1 M Tris, 1% Triton X-114, 10 mM EDTA, 1 mM PMSF, 10 μg/ml Trasylol, pH 8.1, at 0°C. The lysate was clarified by centrifugation, incubated for 10 min at 37°C to induce the phase separation, resuspended at 0°C, re-incubated at 37°C and finally suspended in 2 ml 0.1 M Tris with 0.5% CHAPS to avoid temperature-induced phase separation.

3. RESULTS

The B-cell enriched fraction bound specifically 3.7 ± 0.3% (S.D., $n = 3$) of 5 pM [^{125}I]DFP-uPA (10^6 cells/ml, 16 h at 4°C), as compared with 11.4 ± 0.8% for monocytes and 3.2 ± 0.3% for polymorphonuclear leucocytes. Fig. 1A shows by FACS analysis that about half of the NK (CD16+) cells present in the B-cell enriched fraction were receptor-bearing. None of the B-cells (CD20+) were receptor-positive (not shown). Analogous experiments also revealed receptor heterogeneity among monocytes as previously shown by autoradiography [9]. Fig. 1B shows that the receptor-positive NK cells on average were more granulated than the

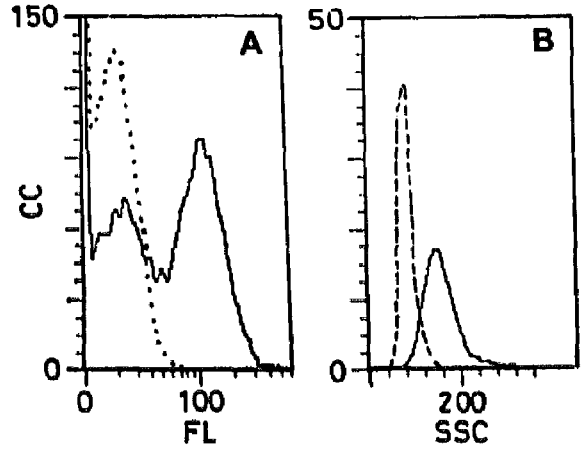

Fig. 1. Binding of biotin-uPA to NK cells. Blood mononuclear cells were incubated for 16 h at 4°C with either 200 pM biotin-uPA alone or plus 200 nM unlabeled uPA, followed by the addition of streptavidin–phycoerythrin and FITC-conjungated antibodies against the CD16 antigen. The gate was set to select CD16+ (NK) cells. (A) The abscissa shows cell-bound biotin-uPA expressed as the fluorescence signal (FL) and the ordinate shows the cell counts (CC): solid line, 200 pM biotin-uPA alone; dotted line, biotin-uPA plus 200 nM unlabeled uPA. (B) The abscissa shows granulation expressed as side scatter (SSC): the receptor-positive CD16+ population is shown with the solid line and the receptor-negative population by the broken line.

receptor-negative cells. Similar analysis using forward scatter (a measure of cell size) showed a larger average size of the receptor-positive cells. Thus, the NK cells able to bind uPA may be classified morphologically as large granular lymphocytes (LGL), and they appear to account for the uPA binding of the B-cell fraction.

The T-cell fraction exhibited <1% binding of [^{125}I]DFP-uPA; the cultured cell line Jurkat was receptor-negative (not shown). FACT analysis (Fig. 2A) revealed a small subset of receptor-positive blood-derived T-cells. Fig. 2B shows that culturing of the T-cell fraction with IL-2 resulted in a population of largely (70–80%) receptor-bearing CD3+ cells. The same result (not shown) was obtained when non-adherent blood mononuclear cells were cultured with IL-2. Culturing peripheral blood mononuclear cells for 5 days with purified protein derivative (PPD), which generated >95% CD3+ T-cells, increased binding to 4.7 ± 0.1% (10^6 cells/ml) as compared to 5.9 ± 0.3% with IL-2. The PPD effect was maximal by 9 to 10 days after a lag time of at least 3 days. Long-term incubation with PPD is known to induce IL-2 secretion by T-helper cells, and the effect is therefore most likely caused by this cytokine.

IL-2-activated T-cells, like those illustrated in Fig. 2B, caused lysis of Daudi cells and could therefore be classified as lymphokine-activated killer (LAK) cells. This lysis seemed independent of uPAR occupancy since incubation with 10-1000 pM catalytically active uPA for up to 24 h had no effect (data not shown).

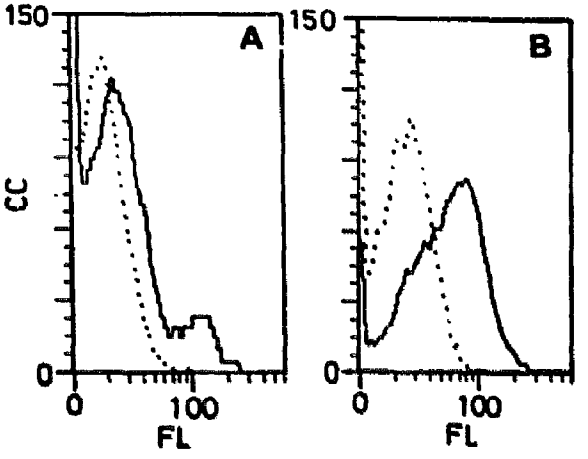

Fig. 2. Binding of biotin-uPA to T-cells and T-cell-derived LAK cells. Cells were incubated as described in the legend to Fig. 1 except that FITC-conjugated anti-CD3 antibodies were used. Abscissa and ordinate are as in Fig. 1A. (A) Non-adherent blood mononuclear cells with the gate set to select T-cells (CD3+); solid line, 200 pM biotin-uPA alone; dotted line, 200 pM biotin uPA plus 200 nM unlabeled uPA. (B) LAK cells. No difference was observed whether or not the cells were gated for CD3; line types are as in A.

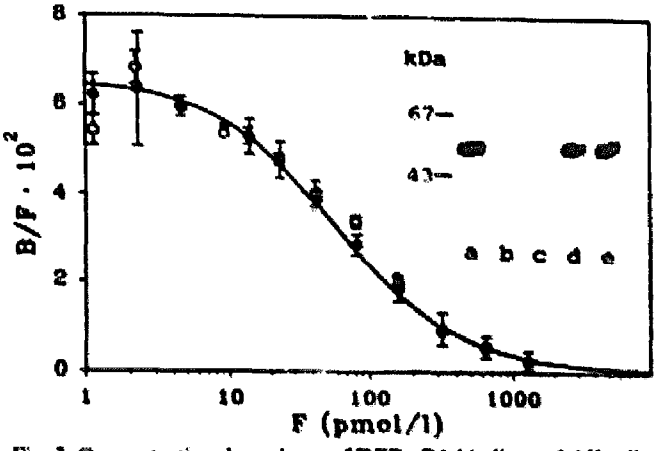

Fig. 3. Concentration dependence of DFP-uPA binding to LAK cells. The cells (1.0×10^6/ml) were incubated at 4°C for 16 h, i.e. at binding plateu. Ligands were 1 pM [^{125}I]DFP-uPA (●) or [^{125}I]uPA:PAI-1 (○) plus unlabeled DFP-uPA to give the indicated total concentrations. The ordinate is the concentration ratio bound/free ligand. The B/F value obtained with tracer plus 100 nM unlabeled DFP-uPA (0.2×10^{-2}) was subtracted from all experimental values. The curve is computed according to the equation: $B/F = R_0/(K_d + F)$, where K_d is the apparent dissociation constant and R_0 the receptor concentration. K_d was calculated as 60 pM, R_0 as 3.7 pM, corresponding to about 2,300 receptors per cell. The points are mean values of triplicates ± 1 S.D. (Inset) [^{125}I]DFP-uPA blotted onto uPAR. Triton X-114 detergent phase (20 μl) was subjected to SDS-PAGE (12% polyacrylamide) and transferred to Immobilon membrane by electroblotting. After blocking (2 h at 22°C in 50 mM Tris, 150 mM NaCl, 2 mM $CaCl_2$, 3% defatted milk powder, 0.05% Tween 20, pH 8.1) and washing, incubation was performed with [^{125}I]DFP-uPA for 5 h followed by autoradiography: lanes a–d, LAK cells; lane e, monocytes. Lanes a and c, 50 pM [^{125}I]DFP-uPA alone; lane b, 50 pM [^{125}I]DFP-uPA plus 100 nM unlabeled uPA; lane c, 50 pM [^{125}I]DFP-uPA plus 100 μg/ml monoclonal antibody directed against the receptor binding domain of uPA; lane d, 50 pM [^{125}I]uPA plus monoclonal antibody against PAI-1.

Initial binding experiments with LAK cells showed no effect of acid wash (pH 3.0), suggesting negligible endogenous occupancy of uPAR. Culture medium and cell extracts did not contain detectable levels of uPA, as determined by ELISA and zymography, suggesting lack of uPA synthesis. Fig. 3 shows an apparent K_d of about 60 pM at 4°C, and the same binding curve was obtained when using labeled uPA:PAI-1 complex. Analogous experiments (not demonstrated) at 37°C showed an apparent K_d of about 150 pM. Ligand blotting revealed an approximately 50 kDa uPAR in LAK cells as in monocytes (Fig. 3, inset) in agreement with uPAR purified from U937 cells [15]. Binding to the receptor protein was blocked by excess unlabeled uPA and inhibited by the monoclonal antibody directed against the receptor-binding domain of uPA, but not by antibody directed against PAI-1.

Pretreatment with 1 U/ml phosphatidylinositol-phospholipase C (1 h, 37°C) reduced binding more than 90% (not shown), suggesting almost complete glycolipid anchoring of uPAR, whereas binding was reduced only 50–60% in monocytes as reported previously for U937 cells [2]. The following results were as reported for monocytes [9]: binding of DFP-uPA, pro-uPA, catalytically active uPA and uPA:PAI-1 complexes were of the same magnitude; tissue type plasminogen activator, alone or in complex with PAI-1, did not inhibit binding of [^{125}I]DFP-uPA.

Fig. 4A shows that uPAR-bound-[^{125}I]DFP-uPA as well as [^{125}I]uPA:PAI-1 dissociated almost entirely as the intact tracers at 37°C in LAK cells. In contrast, as shown in Fig. 4B, appreciable receptor-mediated degradation was observed in blood-derived monocytes: 20% the [^{125}I]DFP-uPA and 60% of the [^{125}I]uPA:PAI-1, that had dissociated to the medium by 4 h, were low molecular weight degradation products.

4. DISCUSSION

This communication demonstrates uPAR in subsets of NK cells (CD16+) and T-cells (CD3+). Both cell classes secrete HuTSP-1, an effective activator of pro-uPA [3,4]. We find that uPA binding to the B-cell enriched blood cell fraction is largely accounted for by the subset of NK cells characterized as LGL, whereas B-cells (CD20+) are u-PAR negative. LGL account for the NK activity in blood [16], secrete pro-uPA [17] and are able to migrate in contrast to B-cells [18]. Future studies should show whether the secretion of HuTSP-1 in NK cells is also accounted for by LGL.

Binding of uPA to blood-derived T-cells has previously escaped notice [10], presumably because only a small fraction is receptor-bearing. The markedly increased expression following culture with IL-2 may qualify uPAR as an activation antigen. Interestingly, the

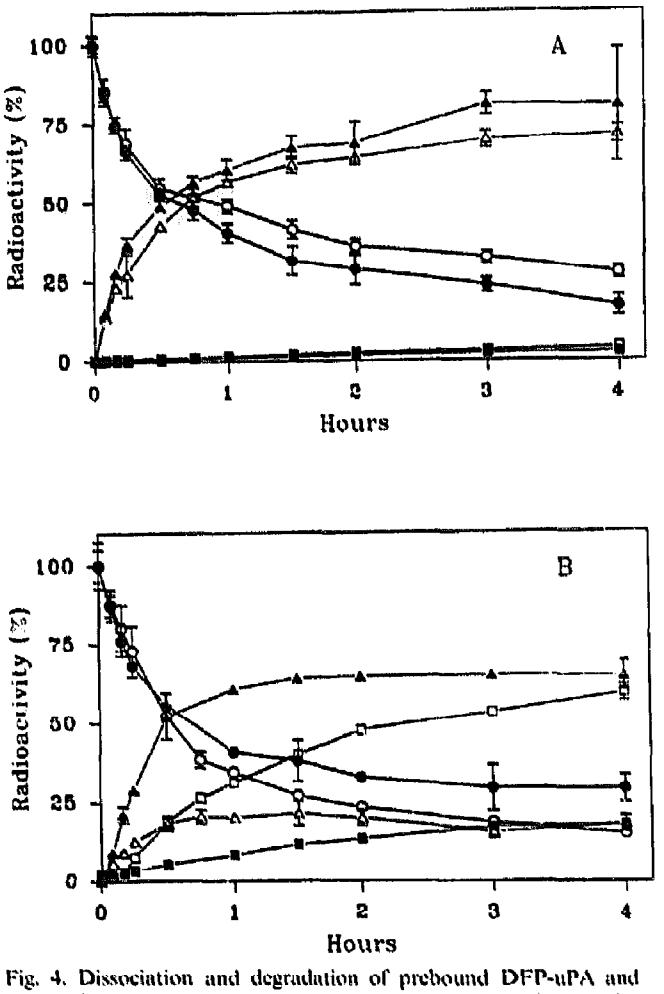

Fig. 4. Dissociation and degradation of prebound DFP-uPA and uPA:PAI-1 in LAK cells (A) and monocytes (B). The cells were incubated for 16 h at 4°C with 10 pM [^{125}I]DFP-uPA (filled symbols) or [^{125}I]uPA:PAI-1 (open symbols), washed and transferred to 37°C (time zero, cell-associated radioactivity set at 100%). Radioactivity remaining uPAR-bound (●, ○); radio-activity appearing in the medium as trichloroacetic acid-soluble (■, □) or -precipitable (▲, △) material. Degradation of the tracers was negligible in both LAK-cell and monocyte-conditioned medium, and dissociated acid-precipitable material was intact DFP-uPA or uPA:PAI-1 as judged by re-binding to fresh cells and SDS-PAGE. The results are means of triplicates ± 1 S.D.

Mo3 activation antigen expressed on the plasma membrane of human mononuclear phagocytes has recently been identified as uPAR [19].

uPAR of IL-2-activated T-cells did not mediate degradation of uPA:PAI-1, even though LAK cells internalize and degrade receptor-bound TNF-α [20]. This is in contrast to the results on monocytes with uPA:PAI-1 and previous results [21] with uPA:plasminogen activator inhibitor type-2 complexes. Since treatment with phosphatidylinositol-phospholipase C removed nearly all uPA binding from LAK cells, but only 50–60% from monocytes, we thought that glycolipid-anchored uPAR might be unable to undergo endocytosis. However, this explanation is unlikely since the same fraction of monocyte-bound uPA was degraded whether or not the cells were pretreated with phospholipase C (unpublished observation). In any event, surface-expressed uPAR in LAK cells appears non-susceptible to the down-regulation induced by uPA:inhibitor complexes in monocytes.

We were unable to detect synthesis of pro-uPA in LAK cells, even though they can extravasate and migrate [11], and they may therefore rely on binding of exogenous pro-uPA. The blood concentration of pro-uPA/uPA is about 15–25 pM [22] implying a receptor occupancy of about 20% (app. K_d ca. 150 pM at 37°C), and higher occupancies of pro-uPA are expected in the neighbourhood of macrophages. Future studies should show whether the population of activated T-cells rich in uPAR is also the population secreting HuTSP-1. If so, this may provide an efficient autocrine mechanism for activation of cell-associated pro-uPA and activation of plasminogen as one of the events facilitating extravasation and migration.

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